



## Original Clinical Research

### Prevalence of Extended Spectrum Beta Lactamase (ESBL<sub>S</sub>) Mediated Resistance among *E. coli* and *Kl. Pneumoniae* in Clinical Samples Taken from Patients Attending in Tertiary Care Hospital, in DMCH, Laheriasarai, Bihar

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#### Abstract

**Objective:** The aim of present study was to determine the prevalence of Extended Spectrum Beta Lactamase (ESBL<sub>S</sub>) mediated resistance among *Escherichia coli* and *klebsiella pneumoniae* in clinical sample and their Antimicrobial Resistance (AMR) Pattern, and also to compare chromogenic agar and combined disc diffusion method in identification of these enzymes in routine clinical laboratories.

**Materials and Methods:** A total of 2640 patient were referred from different OPD and IPD were included in study. From all the patients 2640 clinical specimens like Urine, Pus, CSF, Sputum, Wound swab, discharge from fistula, peritoneal, aspiration, fluid were received in sterilized container by aseptic methods. Any Medical, surgical and drug history were noted. Isolation and Identification of Micro-organism were carried out by Gram's staining culture character, motility and by standard biochemical procedures according to CLSI guidelines. Antimicrobial susceptibility tests was done by Kirby - baur disc diffusion method. Resistance to at least one of the antibiotics (cephalosporin) was considered as positive in the screening test for possible ESBL production. Isolates of *E. Coli* and *Kl. pneumoniae* that were considered to be positive for ESBL production by screening tests were subjected to combined disc diffusion methods and Isolated were also tested for chromogenic ESBL Agar. All the test were performed according to CLSI 2017 guidelines and all the Media, biochemical reagents and Antimicorbial disc are supplied by Hai Media (Mumbai).

**Result:** Out of 2640 clinical specimens 165 Isolates were isolated. *Escherichia coli* were obtained predominantly from Urine (94.1%) samples, where as *klebsiella pneumoniae*, isolates were obtained from sputum (46.2%), the screening test for ESBL production, 78 (42.47%) isolates were found to be resistant to at least one of the cephalosporins disc. Out of 78 isolates that were positive in the screening test, ESBL production was confirmed by combined Disc diffusion method in 62 (79.48%) isolates. ESBL producers were isolated from Urine (33.78%), sputum (80%) and pus (57.14%). ESBL *E. coli* were predominantly isolated from Urine samples (92%), whereas ESBL-KP were isolated from sputum samples (62.5%), conformation of ESBL positive organism by combined disc diffusion method, *E. coli* and *Klebsiella Spp.*

Shown Maximum ESBLs production in CTX & CEC combination. Out of 78 isolates that were positive in the screening test, ESBL production was confirmed by chromogenic ESBL Agar in 77 (98.71%) isolates.

**Conclusion:** Good infection control practices and antibiotic management interventions are instrumental in preventing the emergence of outbreaks due to ESBL producing isolates, especially in high risk areas such as the medical ICU, pediatric wards and surgical wards. Clinical microbiology laboratories need not only use proper phenotypic testing methods but they also need to implement molecular detection protocols. Furthermore, the vigilant surveillance and appropriate infection control programme must be implemented in each hospital setting.

**Keywords:** *Escherichia coli*, *klebsiella spp*, ESBL, Agar, Antimicrobial agent, prevalence.

## Introduction

The members of the family Enterobacteriaceae are one of the most important bacterial pathogens isolated from clinical isolates. In last few years, bacterial resistance has increased dramatically with plasmid mediated ESBL contributing to this increase worldwide. These plasmids also carry co-resistance genes for other non- $\beta$ -lactam antibiotics. Which is also contributor of limiting the number of effective drugs. To make problems worse, plasmid-mediated ESBL enzymes spread fast among bacteria resulting into nosocomial outbreaks. In India, no any national study has been conducted so far for detection of the prevalence of ESBL production, the prevalence rate varies in different institutions from 6-87%. Since no data on ESBL prevalence in our institute was available, so this study was conducted to look for ESBL prevalence.

## Material and Method

Present study was conducted in the Department of Microbiology, Darbhanga Medical College, Laheriasarai, during the period of January 2016 to December 2017.

A total of 2640 patient were referred from different OPD and IPD were included in study. From all the patients 2640 clinical specimens like Urine, Pus, CSF, Sputum, Wound swab, discharge from fistula, peritoneal, aspiration, fluid were received in sterilized container by aseptic methods. Any Medical surgical and drug history were noted. Isolation and Identification of Micro-organism were carried out by Gram's staining, culture character, motility and by standard biochemical procedures according to CLSI 2017

guidelines. All the Media, biochemical reagents and Antimicrobial disc are supplied by Hai Media (Mumbai).

## Antimicrobial Susceptibility Test

Antimicrobial susceptibility test was done by Kirby-Bauer disc diffusion method. A suspension of the isolated bacteria was prepared in sterile normal saline and its turbidity was matched with 0.5 McFarland standard ( $1-2 \times 10^8$  CFU/ml). Sterile swab was dipped into the inoculum and streaked all over the surface of plate. Disc of ceftazidime, cefotaxime, ceftriaxone, aztreonam, cefpodoxime were placed over the inoculated plate and incubated at  $37^{\circ}$  C for 16-18hrs.

According to the Clinical and Laboratory Standards Institute guidelines, isolates were considered as potential ESBL producer if the initial screen tests results were as follows: Zones for Cefpodoxime( $10\mu\text{g}$ ) < 17 mm, Cefazidime ( $30\mu\text{g}$ ) < 22 mm, Aztreonam ( $30\mu\text{g}$ ) < 27 mm, Cefotaxime ( $30\mu\text{g}$ ) < 27 mm and Ceftriaxone ( $30\mu\text{g}$ ) < 25 mm.

Resistance to at least one of the antibiotics was considered as positive in the screening test for possible ESBL production as per 2017 CLSI guidelines

## Combined Disc Diffusion Method

Isolates of *Escherichia coli* and *Klebsiella pneumoniae* that were considered to be positive for ESBL production by the screening test were subjected to the Combined Disc Diffusion Method as recommended by 2017 CLSI guidelines. From the pure cultures of bacteria grown overnight on Mac Conkey agar, a suspension matching 0.5 McFarland standard ( $1-2 \times 10^8$  CFU/ml) was made in sterile normal saline. Using sterile cotton

swab, the bacteria were spread on Mueller Hinton agar to obtain a lawn culture. After allowing the plate to dry, Discs of ceftazidime (30 µg) (CAZ), ceftazidime + clavulanic acid (30/10 µg) (CAC), cefotaxime (30 µg) (CTX), cefotaxime + clavulanic acid (30/10µg) (CEC), cefpodoxime (10µg) (CPD) and cefpodoxime + clavulanic acid (10/5 µg) (CCL) were placed on the surface and the plates were incubated at 37°C for 16-18 hours. Following growth, the diameter of the zones around the discs were measured and recorded. An increase in the zone diameter by  $\geq 5$  mm around the discs containing cephalosporin with clavulanic over the discs containing cephalosporin alone indicated ESBL production.

### Chromogenic ESBL Agar

It was chromogenic screening medium for the selective isolation of ESBL producing organisms. It contains peptone mix and yeast extract, which serves as the carbon and nitrogen sources. Chromogenic mixture was used to differentiate the ESBL producing organisms on the basis of color. ESBL Agar Supplement contains Ceftazidime, Cefotaxime, Ceftriaxone, Aztreonam and Fluconazole helps in inhibition of other contaminating organisms. ESBL producing *Escherichia coli* grow as pink colonies. ESBL producing *Klebsiella pneumoniae* produce bluish green colonies; *Proteus*, *Morganella* and *Providencia* do not utilize any chromogen resulting in colourless colonies.

This medium inoculated with liquid suspension equivalent to 0.5 McFarland turbidity, prepared from isolated colony and incubated at 37°C for 24 hrs and observe.

### Results

Out of 2604 various clinical specimens, a total of 165 isolates of *Escherichia coli* and *Klebsiella pneumoniae* were isolated. Urine (89.7%), sputum (6.1%) and pus (4.2%) were the common samples submitted for culture. Isolates of *Escherichia Coli* were obtained predominantly from urine (94.1%) samples, where as *Klebsiella pneumoniae* isolates

were obtained from sputum (46.2%), urine (38.4%) and pus (15.4%) samples.

**Table 1** shows *Escherichia Coli* and *Klebsiella pneumoniae* from various clinical samples

SAMPLE	ORGANISM		Total
	E.coli	K. pneumoniae	
Urine	143 (94.1%)	5 (38.4%)	148 (89.7%)
Sputum	4 (2.6%)	6 (46.2%)	10 (6.1%)
Pus	5 (3.3%)	2 (15.4%)	7 (4.2%)

Among the patients, from whom the isolates were obtained, 64.84% were female and 35.15% were male. The age of the patients ranged from <1 year to 80 years. The mean age of patients was 40.93 years and the median age was 38 years.

**Table 2** shows Age group of patients from whom the isolates were obtained

AGE	SEX	
	FEMALE	MALE
< =20	18 (16.8%)	4 (6.9%)
21-40	54 (50.5%)	16 (27.6%)
41-60	24 (22.4%)	17 (29.3%)
61-80	11 (10.3%)	21 (36.2%)

### Screening test for ESBL production:

In the screening test, which involved detection of resistance to five cephalosporin antibiotics (ceftazidime, cefotaxime, cefpodoxime, ceftriaxone and aztreonam), 78 isolates (47.27%) were found to be resistant to at least one of the cephalosporin discs. Resistance to cefotaxime was observed in 76 (97.43%), cefpodoxime in 75 (96.15%), aztreonam in 75 (96.15%), ceftazidime in 70 (89.74%) and ceftriaxone in 65 (83.33%) isolates.

Out of the 152 *Escherichia coli* isolates, 67 (44%) were found to be resistant to at least one of the screening agents. Of the 13 *Klebsiella pneumoniae* isolates, 11 (84.61%) were found to be resistant to at least one of the screening agents.

### Confirmation of ESBL production by Combined Disc Diffusion Method

Out of the 78 isolates that were positive in the screening test, ESBL production was confirmed by Combined Disc Diffusion Method in 62 isolates (79.48 %). In 67 isolates of *Escherichia coli* that were positive in the screening test, 51

(76.11%) were phenotypically confirmed as ESBL producers. Similarly, in the 11 *Klebsiella pneumoniae* isolates that were positive in the screening test, all isolates were identified as ESBL producers.

**Table 3** shows Isolates detected as ESBL producers by Combined Disc Diffusion Method

ORGANISM	RESULT		
	ESBL	NON-ESBL	UNKNOWN
Esch. coli	51	85	16
Kleb. Pneumoniae	11	2	0

ESBL producers were isolated from urine (33.78%), sputum (80%) and pus (57.14%). ESBL-EC were predominantly isolated from urine samples (92%), whereas ESBL-KP were isolated predominantly from sputum samples (62.5%), pus (50%) and urine (8%).

**Table 4** shows Distribution of ESBL producers according to the clinical samples

RESULT		SAMPLE			TOTAL
		URINE	PUS	SPUTUM	
ESBL	EC	46	2	3	51
	KP	4	2	5	11
N-ESBL	EC	82	2	1	85
	KP	1	0	1	2
UNKNOWN	EC	15	1	0	16

ESBLs were detected in 54 (87.09%) isolates by using CTX-CEC discs and in 34 (54.83%) isolates by using CAZ-CAC discs. Combinations of both CTX-CEC and CAZ-CAC discs were able to detect ESBL production in 26 (41.93%) of isolates tested.

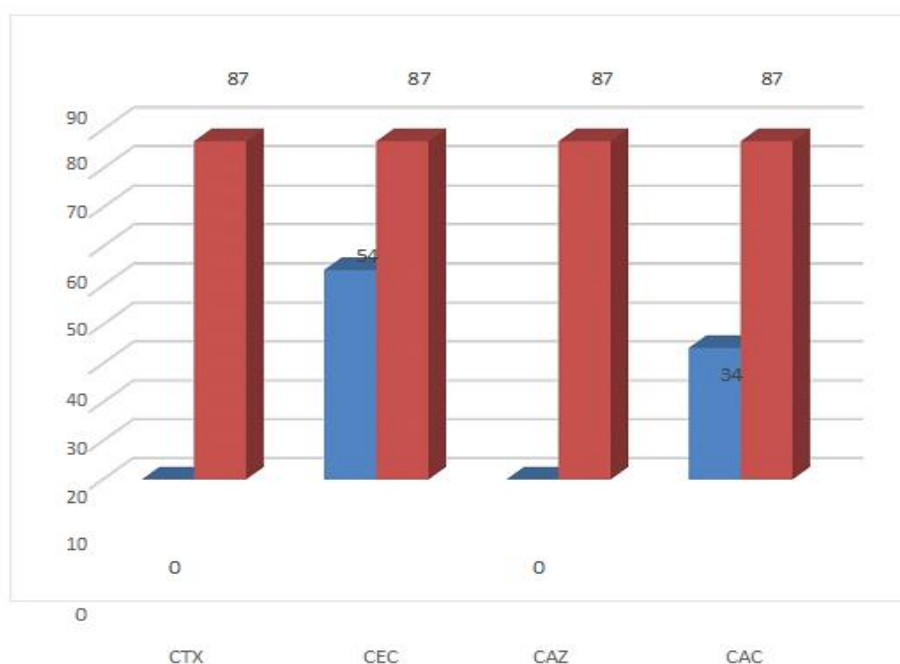
While 28 (45.16%) isolates were detected by CTX-CEC discs only, 8 (12.90%) isolates were detected by CAZ-CAC discs only.

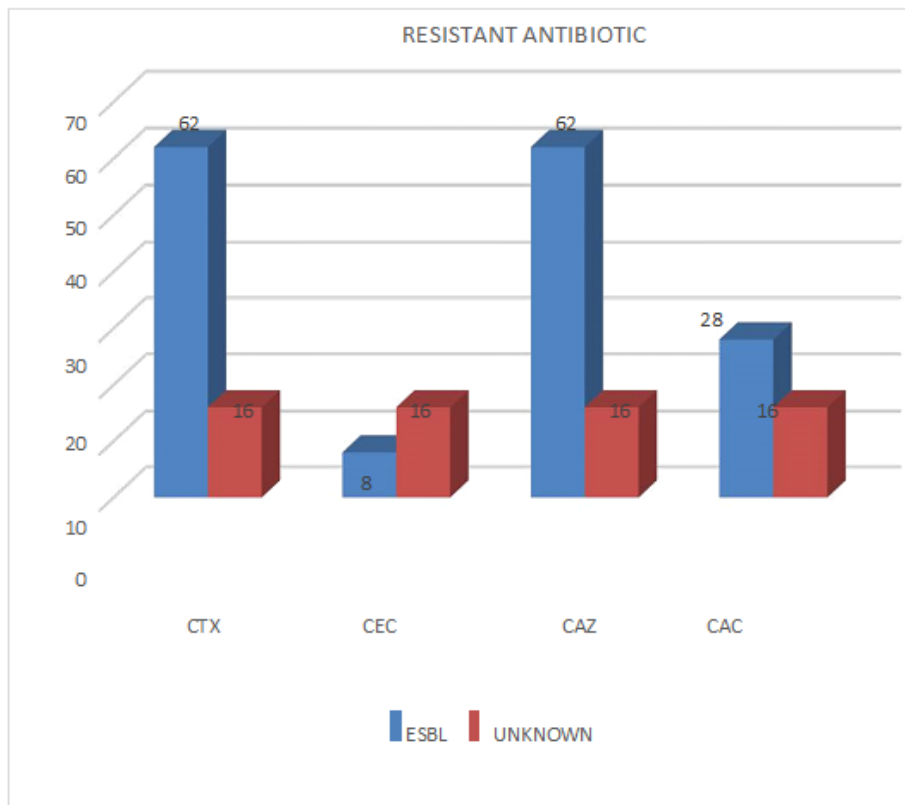
**Table-5**

ANTIBIOTIC DISCS	CTX		CEC		CAZ		CAC	
	R	S	R	S	R	S	R	S
ESBL	62	0	8	54	62	0	28	34
N-ESBL	0	87	0	87	0	87	0	87
UNKNOWN	16	0	16	0	16	0	16	0

Table 5 Showed phenotypic confirmation of ESBL positive bacteria by Combined Disc Diffusion Method by using two combinations, cefotaxime alone and with the combination of clavulanic acid (CTX/CEC) and ceftazidime alone and with the combination of clavulanic acid (CAZ/CAC). Most of the bacteria showed ESBL positive by both combination (CTX/CEC and CAZ/CAC). *Escherichia coli* and *Klebsiella spp.* showed maximum ESBLs production in CTX/CEC combination. Both the CTX/CEC and CAZ/CAC methods were statistically significant.

**Sensitive Antibiotic**





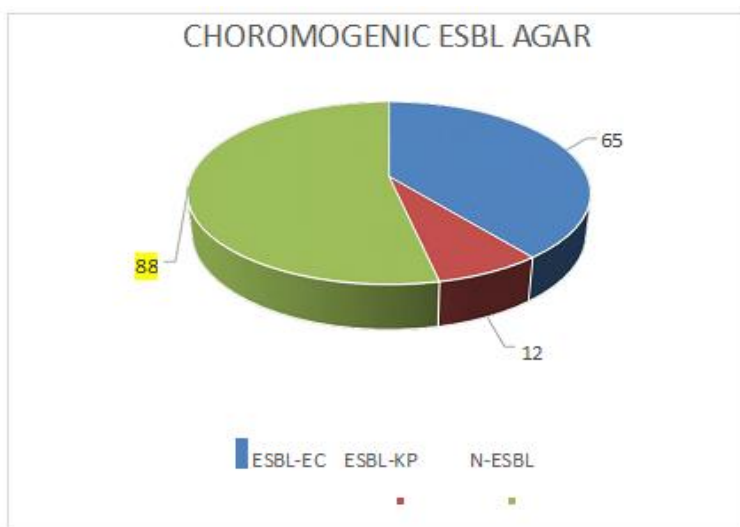
**Chromogenic ESBL Agar**

Out of the 78 isolates that were positive in the screening test, ESBL production was confirmed by Chromogenic ESBL Agar in 77 (98.71 %) isolates. In the 67 isolates of *Escherichia Coli*, that were positive in the screening test, 65 (84.41%) were phenotypically confirmed as ESBL

producers. Similarly, of the 12 (15.58%) *Klebsiella pneumoniae* isolates were identified as ESBL producers.

**Table-7**

ESBL RESULT	CHROMOGENIC ESBL AGAR		TOTAL
	N	P	
ESBL-EC	0	65	65
ESBL-KP	0	12	12
N-ESBL	88	0	88



**Comparison between combined disc diffusion Method and Chromogenic method**

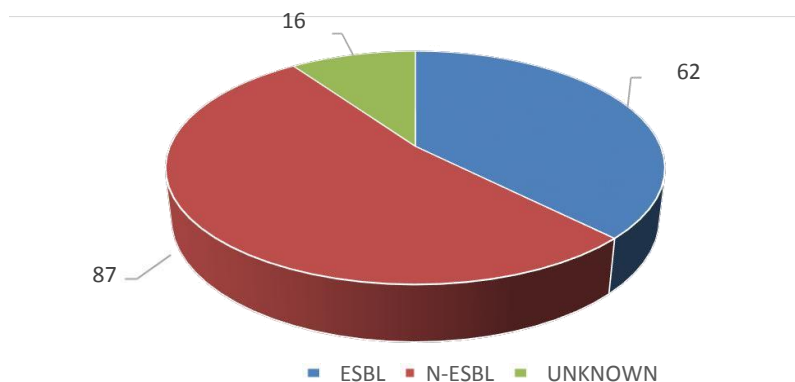
By pooling the results of two confirmatory tests (Combined disc diffusion method and

Chromogenic method). 77 (46.66%) isolates were found to be ESBL positive (Table 7).

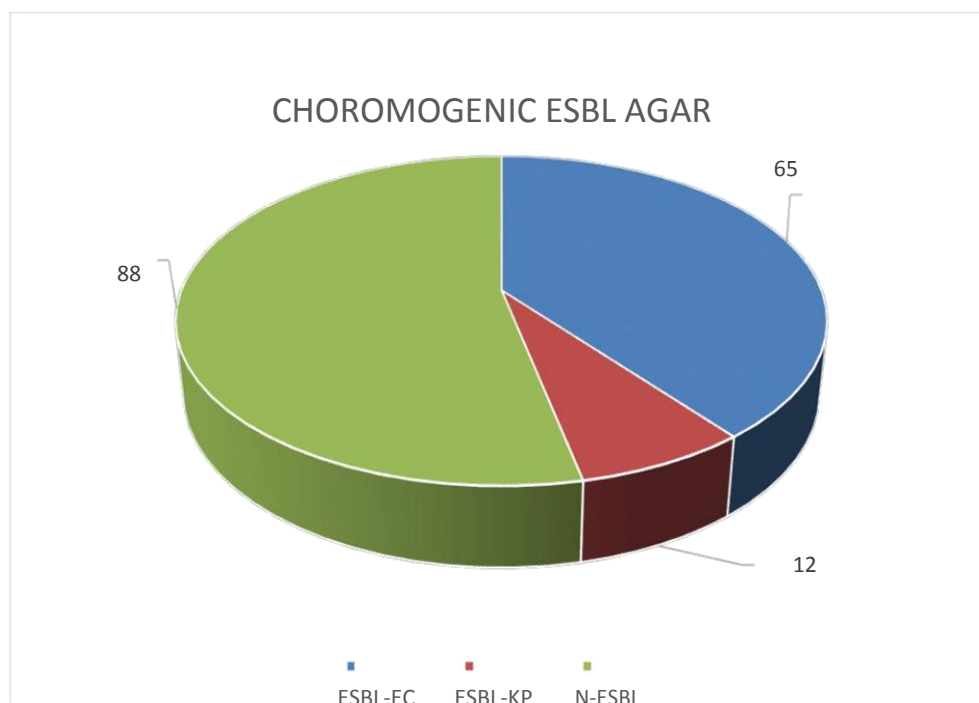
**Table 8** shows Comparison between combined disc diffusion method and Chromogenic method

	Combined Disc Diffusion Method		Chromogenic Agar	
	EC	KP	EC	KP
<b>ESBL</b>	<b>51</b>	<b>11</b>	<b>65</b>	<b>12</b>
<b>N-ESBL</b>	<b>85</b>	<b>2</b>	<b>86</b>	<b>2</b>
<b>UNKNOWN</b>	<b>16</b>	<b>0</b>	<b>0</b>	<b>0</b>
<b>TOTAL</b>	<b>152</b>	<b>13</b>	<b>151</b>	<b>14</b>

COMBINED DISC DIFFUSION METHOD



CHOROMOGENIC ESBL AGAR



**Discussion**

Exposure to hospital environment especially ICUs is a major risk factor for carriage of MDR bacteria especially in resource poor settings where hospitals can have high infection rates. A multitude of factors including poor infrastructure of hospitals, low compliance with hand-hygiene,

heavy workload with understaffing, over crowding, lack of or poorly functioning infection control programme contribute to the problem. Majority of the clinical samples yielding the isolates used in this study came from female patients (64.84%) and most patients from either gender belonged to the age group of 1-80 years.

Urine (89.7%), sputum (6.1%) and pus (4.2%) formed the bulk of the samples that yielded cultures of *Escherichia coli* and *Klebsiella pneumoniae*. *Escherichia coli* isolates were predominantly isolated from urine samples (94.1%) whereas *Klebsiella pneumoniae* isolates were predominantly isolated from sputum (46.2%), urine (38.4%) and pus samples (15.4%). Comparatively more numbers of *Klebsiella pneumoniae* isolates than *Escherichia coli* were recovered from sputum samples. In 2009, CLSI introduced two-step procedure for the detection of ESBL producers. In the first step, isolates of *Escherichia coli* and *Klebsiella pneumoniae* should be screened for resistance to one or more of third-generation indicator cephalosporin (ceftriaxone, cefotaxime, ceftazidime, cefpodoxime or aztreonam). Since ESBLs vary in their hydrolysis of these cephalosporins as substrates, resistance to at least one of them was considered as positive in the screening test.

In the present study, out of 165 clinical isolates 67 (40.6%) of *Escherichia coli* and 11 (6.66%) of *Klebsiella pneumoniae* were found to be positive for possible production of ESBLs in the screening test. The screening test involved ceftriaxone, cefotaxime, ceftazidime, cefpodoxime and aztreonam discs. In the pilot study, the cefpodoxime disc did not meet the expected quality standard and therefore could not be included. False positive screening results with cefpodoxime too have been documented, which have been attributed to a variety of mechanisms including hyperproduction of TEM-1 beta-lactamase, production of OXA-30 beta-lactamase and elevated chromosomal AmpC beta-lactamase production.

The EUCAST guidelines mentions that cefpodoxime is less specific than the combination of cefotaxime, ceftriaxone and ceftazidime for screening ESBLs.

There are some disadvantages associated with the screening test. The two-step detection process adds to the increased cost and delay in reporting results. It is impractical for laboratories that

receive large number of samples or in situations where patients are suffering from severe infections. Although the sensitivity of screening test is high, its specificity is low because a positive test does not necessarily indicate ESBL production alone. Production of other beta-lactamases can also give a positive screening test. In the second step recommended by CLSI, isolates that are positive in the screening test are confirmed for ESBL production by clavulanic acid based test. Although ESBLs are inhibited by clavulanic acid, sulbactam and tazobactam, most phenotypic methods are based on clavulanic acid. In this study, both ceftazidime, ceftazidime+clavulanic acid (CAZ/CAC) and cefotaxime, cefotaxime+clavulanic acid (CTX/CEC) discs were used as per CLSI protocols. CTX/CEC discs were able to detect ESBL production in 54 (87.09%) of isolates that were positive in the screening test. CAZ/CAC discs were able to detect ESBLs in only 34 (54.83%) of isolates, suggesting that CTX/CEC discs were more sensitive in detecting ESBLs. However, if CTX/CEC discs alone were to be used, eight ESBL producers would have been missed. Similarly, 28 ESBL producers would have been missed if only CAZ/CAC discs were to be used.

CLSI recommends that both cefotaxime and ceftazidime, alone and in combination with clavulanic acid must be used for detection of ESBLs. The reason for such recommendation is due to the fact that ESBLs vary in their ability to hydrolyze third generation cephalosporins as substrates. Cefotaximase type ESBLs would be missed if only ceftazidime were to be used and ceftazidimases would be missed if only cefotaxime were to be used. Most CTX-M type ESBLs except those hydrolyze ceftazidime as well, would be missed if only ceftazidime were to be used. Yet, several studies from India have used either of them in their disc diffusion assays. Thus, the CLSI Guidelines on using both disc combinations is imperative.

Out of the 78 isolates that were positive in the screening test in this study, 62 (79.48%) isolates

could be phenotypically confirmed as ESBL producers by the Combined Disc Diffusion method. In 67 isolates of *Escherichia coli* that were positive in the screening test, 51 (76.11%) were phenotypically confirmed as ESBL producers. Similarly, in the 11 *Klebsiella pneumoniae* isolates that were positive in the screening test, all isolates were identified as ESBL producers.

Out of the 78 isolates that were positive in the screening test, ESBL production was confirmed by Chromogenic ESBL Agar in 77 (98.71 %) isolates. In the 67 isolates of *Escherichia coli*, that were positive in the screening test, 65 (84.41%) were phenotypically confirmed as ESBL producers. Similarly, of the 12 (15.58%) *Klebsiella pneumoniae* isolates were identified as ESBL producers.

Garima et al. studied the occurrence of ESBL producers among the Enterobacteriaceae was 25.67 % .

Manoj et al. studied among *Escherichia coli* isolates, ESBL production was observed in 81.2%, 80.0%, 76.8% and 75.2% of 250 isolates by ESBL detection kit, double disc synergy test (DDST), Etest for ceftazidime and cefotaxime respectively, Among *Klebsiella* isolates, ESBL production was observed in 63.3%, 62.2% and 61.0% of 267 isolates by Etest, ESBL detection kit and double disc synergy test (DDST).

Shobha et al. detected *Escherichia coli* (32%) and *Klebsiella* (37%) isolates to be ESBL producers with the screening test, *Escherichia coli* (35%) and *Klebsiella* (41%) with the phenotypic confirmatory test using modified double disc method.

Jitsurong *et al.* detected 5.1% of *Escherichia coli* isolate and 44.4% of *Klebsiella pneumoniae* isolates as ESBL producers with screening disc diffusion test, combination disc test and Etest.

Shah et al. studied the relation of ESBL-producing Enterobacteriaceae with respect to age and gender and reported more ESBL-positive isolates in males (65.33%) than females (34.67%). Similar

findings were observed in the present study by combined disc diffusion method.

On comparing with present study, ESBL production in urine samples (33.78%), pus samples (57.14%) and sputum samples (80%), Uma devi S et al exudates (66.7%), urine (75%) was noted, Kaur M et al, ESBL production in pus samples (51.37%), followed by urine samples (45.63%).

### Conclusion

In the present study, the occurrence of ESBL was higher in hospitalized patients as compared to outpatients which is statistically significant. The reason for which may be lack of hygiene, cross infection among the large populations, lack of counter availability of antibiotics, lack of awareness and drug administration from quacks who frequently abuse antibiotics .

New technologies such as molecular techniques and modified mass spectrometry technique (Matrix Assisted Light Desorption Ionization Time-of-Flight) are being suggested as quicker alternatives for routine laboratory diagnosis. However these are available only in research facilities and are still new in their development. Hence, routine detection of ESBLs by conventional methods should be done in every laboratory where molecular methods cannot be prefer.

The Ethical committee clearance has been obtained from our Institution.

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